



Plant growth-promoting characteristics of root fungal endophytes isolated from a traditional Cordillera rice landrace

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Abstract

This study was conducted to isolate and characterize the plant growth-promoting potential of fungal endophytes from the roots of Diket red, a traditional rice plant from the Cordillera, Northern Luzon. Eighteen morphospecies of filamentous endophytes were isolated of which twelve isolates were successfully identified to the species level. These isolates were identified as *Aspergillus versicolor*, *Aspergillus* sp., *Chaetosphaeria* sp., *Cladosporium cladosporioides*, *Hypocrea lixii*, *Microascus murinus*, and *Trichoderma harzianum*. The identified twelve isolates were selected to screen *in vitro* for their plant growth-promoting characteristics, and evaluated *in vivo* for their beneficial effects on seedling vigor and early seedling growth. Isolate PPL14 produced the highest IAA at 55.5 $\mu\text{g ml}^{-1}$ and *M. murinus* PPL10 produced the highest amount of IAA at 3.73 $\mu\text{g IAA mg}^{-1}$ dry mycelia wt. The seedling vigor assay and *in vivo* plant growth promotion bioassay indicated overall positive effects of culture filtrate (CF) application of the endophyte isolates. Rice seeds and seedlings grown in aseptic condition and treated with endophyte CFs displayed significantly enhanced levels of germination, seedling vigor, shoot, root, and total plant growth, and biomass compared to non-treated control. Other plant growth-promoting characteristics were also studied including phosphate solubilization, siderophore production, ammonia production, and catalase activity. This study supports the potential use of fungal endophytes as bio-inoculants for plant growth promotion and enhancement of nutrient assimilation of agriculturally important crops.

Key words – 18S rDNA – Endophytic fungi – Plant growth promotion – Rice landrace – Seedling vigor

Introduction

Fungal endophytes refer to fungi existing in diverse types of plant hosts that colonize living plant tissue without causing any immediate visible disease symptoms (Schulz & Boyle 2005). They constitute a major portion of fungal symbionts associated with plants and are usually associated with roots, stems, and leaves (Khan et al. 2012a). These fungi are of great interest because of their significant roles as mutualistic symbionts of plants, rendering positive effects to their hosts by acting as protective mutualists and by providing beneficial secondary metabolites (Mariño et al. 2007). They have also been found to confer beneficial traits to their host plant such as tolerance to abiotic stresses, resistance against pathogen attacks, enhanced nutrient assimilation, and plant growth promotion (Rodriguez et al. 2008, Khan et al. 2012b, Waqas et al. 2012, Lahlali et al. 2014). In recent years,

researches focusing on fungal endophytes have been growing because of their promising potential applications in medicine, pharmaceuticals, and agriculture (Strobel & Daisy 2003, Rai et al. 2014).

Plant growth-promoting (PGP) endophytic fungi are significant in an agriculture point of view since they can be utilized as possible alternative to chemical fertilizers to ensure good crop yields, maintain soil integrity, and preserve long term ecological balance in agro-ecosystems. The increased interest for low-input agriculture has in effect increased the popularity of plant growth-promoting rhizobacteria (PGPR) as bio-inoculant to mobilize soil nutrients and enhance nutrient cycling. On the other hand, endophytic association of fungi with plants occurred for >400 Myr ago as supported by fossil records (Krings et al. 2007). Despite this long symbiosis and over 100 years of research on fungal endophytes, studies regarding the potential applications of PGP fungal endophytes for agricultural purposes have been narrow (Hamayun et al. 2011).

Bioprospecting endophytic fungi from plants growing in high stress environments and harboring great genetic diversity may offer novel untapped sources of microorganisms with beneficial traits that could be useful for crop growth improvement. For instance, colonization with endophytes isolated from high stress environments conferred drought, salinity, and cold tolerance to the rice plant (Redman et al. 2011). Wild crop relatives that adapted to suboptimal conditions are good sources of genetic traits for tolerance to abiotic stress, and exploration of fungal endophytes from these wild relatives offer an opportunity to unravel the mechanisms of habitat adaptation in high stress and suboptimal environments (Yokoya et al. 2017).

A traditional Cordillera rice variety or landrace such as *Diket red* was the preferred source of endophytic fungi due to the reported unmatched quality traits of rice landraces including rich genetic diversity, adaptation to wide agro-ecological niches, and resistance to insects and pathogens (Ram et al. 2007). Rice landraces in the Cordillera highlands of Northern Philippines are frequently exposed to high stress conditions because of the environment where they are being cultivated. The Cordillera region has a mountainous terrain, characterized by inherently cold temperatures due to high altitude and high surface water run-off due to its steep slopes. Frequent exposure to these stresses makes these rice landraces develop resistance to cold temperature and drought. Moreover, the soil tends to be rich in toxic elements such as aluminum and manganese and lacking in necessary minerals such as iron and phosphorus (Chang & Vergara 1975). The enhanced adaptation and growth of rice landraces under adverse environment is a feature that could be attributed to symbiosis of the plant with endophytic fungi (Rodriguez et al. 2004). Fungal endophytes associated with *Diket red* therefore, may play key roles that could contribute to the adaptive traits of this landrace.

In this work, a selection of plant growth-promoting characteristics of the fungal endophytes isolated from the roots of *Diket red*, a traditional rice landrace, was reported. This study further assessed the bioactive potential of the isolated endophytes for enhancement of rice seedling vigor and plant growth. These assessments can help in understanding the beneficial interactions of the fungal endophytes with their host plant and to suggest possible applications.

Materials & Methods

Indole-3-acetic acid (IAA) production

Endophyte isolates were grown in 50 ml potato dextrose broth (PDB) amended with 0.1% (w/v) L-tryptophan for 7 days in an incubator set at 30°C. After 7 days, the broths were filtered to separate the fungal mycelia from the filtrates. The culture filtrates (CF) were then used for the colorimetric assay of IAA. For the assay, 1 ml of the filtrate was mixed with 2 ml Salkowski reagent (12 g l⁻¹ FeCl₃ and 7.9 M H₂SO₄) based on Glickmann & Dessaux (1995). This was mixed and incubated in the dark for 30 min until complete pink color development. The color intensity of the mixture was quantified by a Shimadzu UV mini 1240 UV-VIS spectrophotometer (Shimadzu, Kyoto, Japan) at 530 nm wavelength. The resulting IAA concentration of the samples were compared with the standard calibration curve obtained from a series of pure IAA standard (Merck, Germany) solutions (5 ppm, 10 ppm, 20 ppm, 50 ppm, 100 ppm; R² = 0.927). The fungal mycelia were oven dried at 60°C for 2 days and weighed for normalizing production of IAA per unit dry mycelia weight. Based

on their phytohormone production, 12 endophyte isolates were selected for further studies i.e. to identify and determine other plant growth-promoting characteristics.

Phosphate solubilization

The phosphate solubilizing capabilities of the isolates were tested on Pikovskaya's agar (Titan Media, India) amended with 0.003% Bromocresol green as indicator. Endophyte isolates were spot inoculated onto the medium and incubated at 28°C for 7 days. The formation of a clear halo zone around the fungal hyphae indicated the ability of the fungi to solubilize inorganic phosphate. The phosphate solubilization index (SI) was calculated using the formula (Vitorino et al. 2012):

$$SI = \frac{\text{Halo diameter}}{\text{Colony diameter}}$$

Siderophore production

Siderophore production was tested using ferric chloride (FeCl₃) test (Neilands 1981, Vala et al. 2006). To determine the catechol nature of the siderophores, 1 ml of 2% FeCl₃ was added to a 1 ml CF and scanned using a spectrophotometer at 495 nm for peak in absorbance, which indicated a positive result. Hydroxamate nature of the siderophores was also done in a similar way, 1-5 ml of 2% FeCl₃ was added to a 1 ml CF and scanned for peak in absorbance between 420-450 nm of the ferrated siderophores.

Ammonia (NH₃) production

The isolates grown in PDA slants were used as inocula and the method of detecting NH₃ production using formulated agar slants described by Hucker & Wall (1922) was employed. The endophyte isolates were grown on agar slants for 7 days at 30°C. After incubation, 1 ml each of 1% phenol solution and NaClO (1% available chlorine) was added to the surface of the slants. These were allowed to stand for 30 min and observed for the appearance of blue color as a positive result.

Catalase test

Isolates grown on PDB incubated at 30°C for 24 h was tested for catalase by placing a small amount of the culture on a glass slide and mixing it with appropriate amount of H₂O₂. Effervescence from the samples indicated a positive result.

Assessment of growth-promoting metabolites

Seed surface sterilization

The rice seeds were surface sterilized with 2.5% NaClO for 30 min and rinsed twice with deionized distilled (Type 1) H₂O. To prevent seed coat GA biosynthesis, 20 ppm paclobutrazol was added to commercial PSB Rc10 rice seeds and incubated in room temperature for 24 h. The treated seeds were washed with Type 1 H₂O twice (Khan et al. 2012b).

Effect on seedling vigor index (SVI)

Sterilized rice seeds were soaked with the endophyte CF for 1 h with gentle agitation. The seeds were germinated in petri plates lined with sterile cotton moistened with distilled H₂O inside an incubator for 8 days at 25°C constant temperature. Shoot length and root length were measured and the germination rate was calculated based from the number of germinated seeds per treatment. The experimental data were compared with rice seedlings treated with autoclaved Type 1 H₂O (negative control) and 20 ppm gibberellic acid (GA) (Sta. Cruz Chem, USA) and 20 ppm IAA (Merck, Germany) standards (positive controls). Three replicates of ten plants each were used and SVI was computed based on the formula given below (Amprayan et al. 2012):

$$\text{Seedling vigor index (SVI)} = \% \text{ Germination} \times (\text{shoot length} + \text{root length})$$

Rice seedling bioassay

To assess *in vivo* plant growth-promoting activity of the endophyte isolates, the method described by Khan et al. (2012b) was used and PSB Rc10, a popular early-maturity rice variety obtained from the Philippine Rice Research Institute (PhilRice), was used as test plant. The sterilized seeds were soaked with Type 1 H₂O until radicle emergence. The germinated seeds were transplanted into sterilized glass growth chambers containing 0.8% water: agar medium. At the two-leaf stage (approx. after 10 days), 20 µl of CF was applied to the shoot apex of the rice seedlings. The glass growth chambers were placed on a fluorescent lighting shelf, and the plants were grown at room temperature (20 ± 2°C). After 7 days, several parameters for plant growth promotion were measured such as plant height, shoot length, and root length. The total plant, shoot, and root dry weights were also recorded and compared with PSB Rc10 rice seedlings treated with autoclaved Type 1 H₂O and PDB (negative controls) and 20 ppm GA and 20 ppm IAA standards (positive controls). This was done in triplicates of five plants each.

Statistical analysis

The data were statistically analyzed by one-way Analysis of Variance (ANOVA) using SPSS® software version 20.0 (SPSS Inc., Chicago, USA). The significant differences of the mean values were compared by Duncan's Multiple Range Test (DMRT) at $P \leq 0.05$.

Results

Molecular identification and phylogenetic analysis

A total of 18 morphospecies of fungal endophytes selected on the basis of colony color and morphology were isolated from the roots of *Diket red*. Analysis of the amplified ITS region on the 18S rDNA queried to the GenBank database resulted to the successful identification of 12 fungal endophytes (Table 1). The isolates were identified as two *Cladosporium cladosporioides* (PPL2 and PPL3), two *Microascus murinus* (PPL10 and PPL18), five *Aspergillus versicolor* (PPL5, PPL7, PPL15, PPL16 and PPL17), *Trichoderma harzianum* (PPL6), *Hypocrea lixii* (PPL8), and *Chaetosphaeria* sp. (PPL12). The isolate PPL9 was identified as *Aspergillus caesiellus* with only 72% support from the Genbank query, thus its identification is reduced to unidentified Ascomycete.

The 12 isolate sequences with >85% sequence similarity as determined by BLASTn search were aligned and the phylogenetic tree was constructed using maximum likelihood method with 1,000 bootstrap replications in MEGA 6 (Fig. 1). The result of the phylogenetic analysis showed distinct clustering of the isolates. One cluster exclusively contained *Cladosporium spp.* and the other cluster contained *Aspergillus spp.* The third cluster contained the isolates *Trichoderma harzianum*, *Hypocrea lixii*, *Chaetosphaeria* sp., and *Microascus murinus*.

Assay for indole-3-acetic acid production

Colorimetric assay for IAA showed that all isolates were able to produce IAA (Fig. 2). The highest IAA produced was 55.5 µg ml⁻¹ by the isolate PPL14 (Fig. 2A). Other notable isolates based on the amount of produced IAA per ml were *H. lixii* PPL8 (45.83 µg ml⁻¹), *M. murinus* PPL18 (45.78 µg ml⁻¹), *A. versicolor* PPL15 (44.57 µg ml⁻¹), and isolate PPL9 (43.86 µg ml⁻¹). In order to normalize the production of IAA, the weights of mycelia of the fungal endophyte isolates were recorded. Comparison of the different IAA production of the isolates in terms of IAA produced per mg of dry mycelia weight, the isolates *M. murinus* PPL10 and *C. cladosporioides* PPL3 had values which were significantly higher than the other isolates (Fig. 2B). The isolate *M. murinus* PPL10 produced the highest amount of auxin which was 3.73 µg mg⁻¹ and isolate *C. cladosporioides* PPL3 produced 3.66 µg mg⁻¹.

Other plant growth-promoting characteristics

To further describe other plant growth-promoting characteristics of the isolates, the 12 selected isolates were further studied. Their capabilities to produce siderophores, solubilize inorganic

phosphates, produce NH₃, and exhibit catalase activity were tested (Table 2). The FeCl₃ test showed that all fungal endophytes have the ability to produce hydroxamate siderophore as determined by the peak in absorbance between the wavelength 420–450 nm. The catecholate type siderophore was not detected in all of the endophyte CFs since there was no peak in absorbance at 495 nm.

Table 1 Identification of fungal endophyte isolates from the roots of *Diket red* based on genetic analysis of the internal transcribed spacer (ITS) region of the 18S rDNA.

Fungal isolates	NCBI accession number	Sequence Analysis using BLASTn search algorithm		Identification
		Closest NCBI match with accession number	Percentage Homology	
PPL2	KT589836	<i>Cladosporium cladosporioides</i> (HQ671181.1)	99%	<i>Cladosporium cladosporioides</i>
PPL3	KT589837	<i>Cladosporium cladosporioides</i> (KJ589558.1)	99%	<i>Cladosporium cladosporioides</i>
PPL5	KT589838	<i>Aspergillus versicolor</i> (KP027423.1)	99%	<i>Aspergillus versicolor</i>
PPL6	KT589839	<i>Trichoderma harzianum</i> (KM079612.1)	99%	<i>Trichoderma harzianum</i>
PPL7	KT589840	<i>Aspergillus versicolor</i> (KM396917.1)	99%	<i>Aspergillus versicolor</i>
PPL8	KT589841	<i>Hypocrea lixii</i> (JX069199.1)	99%	<i>Hypocrea lixii</i>
PPL9	KT589842	<i>Aspergillus caesiellus</i> (EF652044.1)	72%	Unidentified Ascomycete
PPL10	KT589843	<i>Scopulariopsis murina</i> (KF986441.1)	99%	<i>Microascus murinus</i> *
PPL12	KT589844	<i>Chaetosphaeria tulasneorum</i> (AF178547.2)	89%	<i>Chaetosphaeria</i> sp.
PPL15	KT589845	<i>Aspergillus versicolor</i> (KP081774.1)	99%	<i>Aspergillus versicolor</i>
PPL16	KT589846	<i>Aspergillus versicolor</i> (KM396917.1)	99%	<i>Aspergillus versicolor</i>
PPL17	KT589847	<i>Aspergillus versicolor</i> (KM396917.1)	99%	<i>Aspergillus versicolor</i>
PPL18	KT589848	<i>Scopulariopsis murina</i> (KF986441.1)	99%	<i>Microascus murinus</i> *

*Basionym: *Scopulariopsis murina* (Samson & Klopotek 1972) recently redefined as *Microascus murinus* by Sandoval-Denis et al. (2016)

On the other hand, the phosphate solubilization assay showed that 4 endophyte isolates, namely isolate PPL14 expressed the highest SI value of 1.80 followed by *C. cladosporioides* PPL3, *A. versicolor* PPL15, and *C. cladosporioides* PPL2 (Table 2). These 5 isolates showed positive result by changing the color of the Pikovskaya's agar medium immediately surrounding the colony from blue to yellow. All of the isolates showed positive result in the test for NH₃ production and catalase activity (Table 2). The results showed that *A. versicolor* PPL5, *H. lixii* PPL8, *M. murinus* PPL10, isolate PPL11, isolate PPL14, and *M. murinus* PPL18 produced the highest amount of NH₃ based on their strong blue color reaction.

Seedling vigor index (SVI)

The presence of bioactive compounds produced by the endophyte isolates were tested for their effect on enhancing rice seed germination and vigor. After incubation, noticeable differences in the shoot length of the germinated seeds were observed (Table 3). There was a significant difference among the treatments in terms of shoot length enhancement, with the isolates PPL11 and *Chaetosphaeria* sp. PPL12 significantly promoted shoot elongation when compared to the seedlings

treated with the negative control and IAA. However, the increase in shoot length was not significantly comparable to the effect of GA. In terms of root elongation, *T. harzianum* PPL6, isolate PPL11, and *Chaetosphaeria* sp. PPL12 had the highest effects (Table 3).

The SVI of the rice seedlings treated with the CFs were not significantly different from each other. However, the increase in seedling growth and vigor conferred by the isolate PPL11, *T. harzianum* PPL6, *Chaetosphaeria* sp. PPL12, *A. versicolor* PPL15, and isolate PPL18 were observably higher compared to other isolates and the controls except the GA-treated seeds.

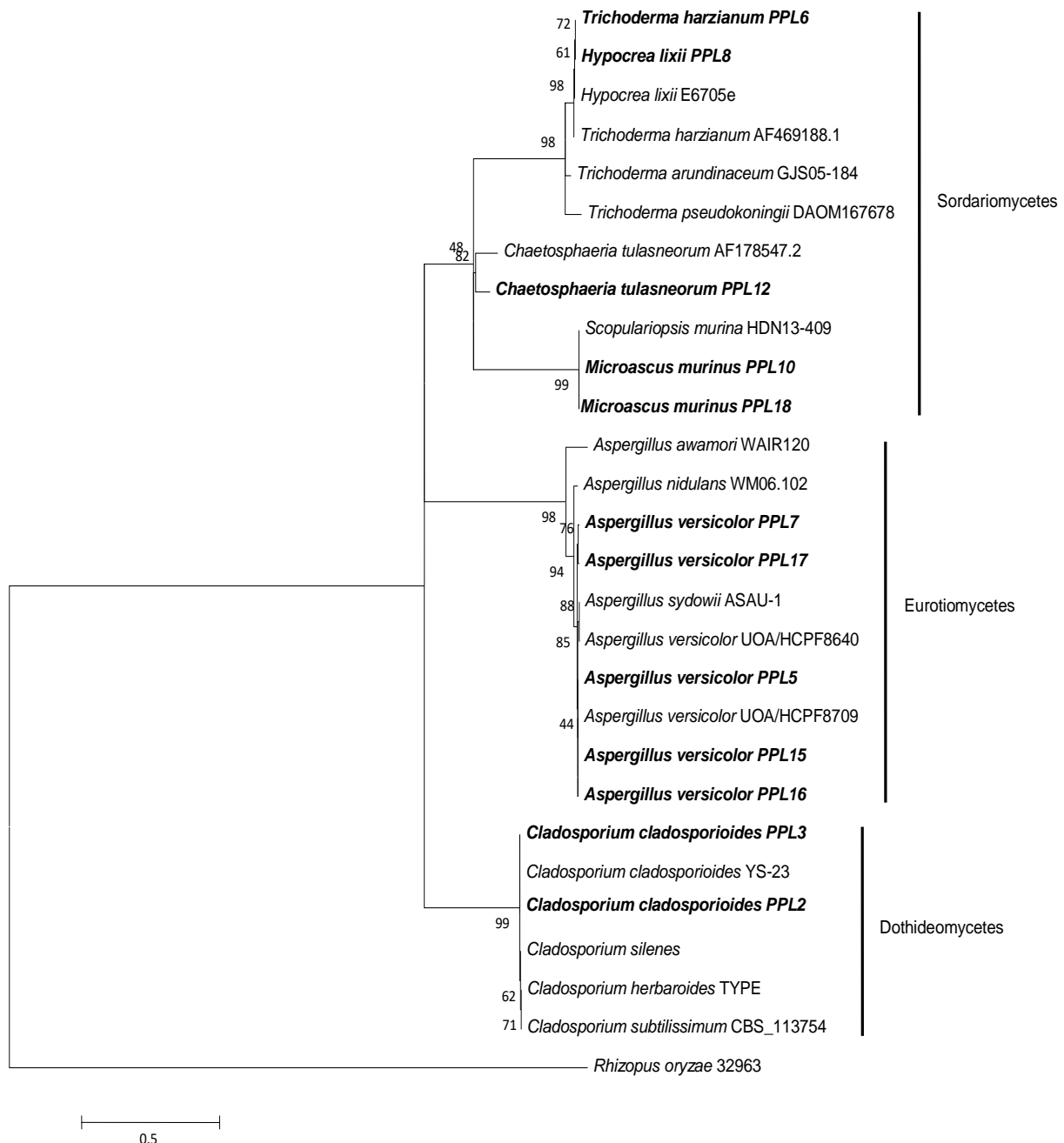


Fig. 1 – Phylogenetic tree from analysis of 18S rDNA sequences of the fungal endophyte isolates from *Diket red* conducted in MEGA 6 using maximum likelihood method. The values next to branch points represent the percent bootstrap support as calculated from 1000 bootstrap replications (Tamura et al. 2013). Isolates with PPL affixed to their names are fungal endophytes obtained from roots of *Diket red* in this study.

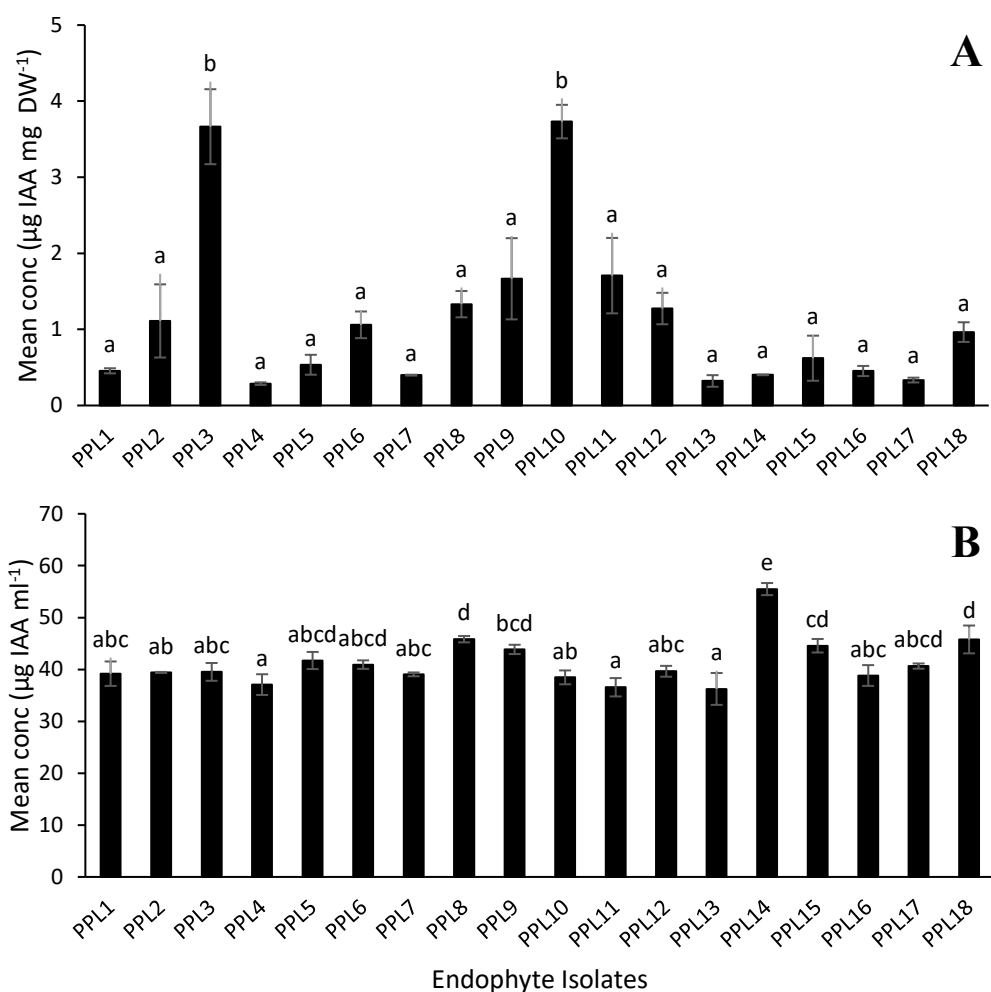


Fig. 2 – IAA production of the endophyte isolates per ml of PDB (A) and per mg dry weight (B) incubated with 0.1% L-tryptophan. Data bars having different letters are significantly different from each other as determined by DMRT ($P \leq 0.05$). The error bars represent SE of the mean ($n = 3$).

Rice seedling bioassay

Promotion of plant growth

The endophyte CFs were assayed on PSB Rc10 rice seedlings to further assess the phytostimulatory effects of bioactive metabolites produced by the endophyte isolates. The influence on plant shoot elongation ranged from 10.13 (negative control dH₂O) to 20.85 cm (positive control GA) (Fig. 3). The isolates *H. lixii* PPL8, *T. harzianum* PPL6, *A. versicolor* PPL15, *M. murinus* PPL10, and the control GA significantly promoted shoot elongation of the rice seedlings. As predicted, the highest shoot growth promotion was from the GA treated rice seedlings, which increased shoot growth to 105.82% compared to dH₂O control and made the seedling shoots appear spindly, a characteristic of GA application. Isolates *H. lixii* PPL8 and *T. harzianum* PPL6 followed GA in significantly increasing shoot growth. The isolate *H. lixii* PPL8 significantly increased shoot growth to 21.62% more than the negative control and 4.85% more than IAA. On the other hand, *T. harzianum* PPL6 promoted shoot growth to 19.15% compared to the negative control and 2.72% compared to IAA. In terms of root elongation, the values ranged from 5.58 (dH₂O) to 6.92 cm (*M. murinus* PPL18) (Fig. 3). All isolates influenced the root elongation of rice seedlings when compared to the negative control. However, *C. cladosporioides* PPL2 demonstrated the least stimulatory effect on root elongation, increasing it to only 2.87% compared to the negative control. On the contrary, *M.*

murinus PPL18 promoted root elongation to 24.01% compared to the negative control, 8.98% compared to the IAA and 4.06% compared to the GA; while, the isolate PPL9 isolate promoted it to 20.43% compared to negative control, 5.83% compared to IAA, and 1.05% compared to the GA. For the total plant height among all the endophyte isolates, *M. murinus* PPL18, *H. lixii* PPL8, *T. harzianum* PPL6, *A. versicolor* PPL15, and isolate PPL14 significantly promoted the overall plant growth of rice seedlings; with the positive control GA having the highest promotion of rice seedling growth (27.48 cm) (Fig. 3). *M. murinus* PPL18 increased seedling plant height by 18.14% compared to dH₂O and 3.20% compared to IAA. On the other hand, *H. lixii* PPL8 and *T. harzianum* PPL6 promoted plant height to 17.57% and 17.00% increase compared to dH₂O, and to 2.71% and 2.21% increase compared to IAA.

Table 2 Other plant growth-promoting characteristics of the isolated fungal endophytes from *Diket* red roots.

Endophyte Isolates	FeCl ₃ test ^a		Phosphate solubilization (SI) ^b	NH ₃ production ^c	Catalase test ^a
	Hydroxamate (Peak 425-450 nm)	Catecholates (Peak 495 nm)			
PPL2	+	–	1.18	+	+
PPL3	+	–	1.58	+	+
PPL5	+	–	0	++	+
PPL6	+	–	0	+	+
PPL8	+	–	0	++	+
PPL9	+	–	0	+	+
PPL10	+	–	0	++	+
PPL11	+	–	0	++	+
PPL12	+	–	0	+	+
PPL14	+	–	1.80	++	+
PPL15	+	–	1.50	+	+
PPL18	+	–	0	++	+

^a + positive, – not detected, ^b Solubilization index (SI) = halo diameter/colony diameter, ^c + positive result, ++ strong positive result

Table 3 Influence of endophyte culture filtrates (CFs) on seed germination and vigor. Values are means of three replicates ± SE*. Means with different letters in the same column are significantly different according to DMRT at $P \leq 0.05$.

Treatment	Germination (%)	Shoot Length (cm)	Root Length (cm)	Vigor Index
dH ₂ O	93.30 ± 3.33	4.67 ± 0.53 ^b	6.37 ± 1.41 ^{ab}	1029.44 ± 155.26 ^{abc}
IAA	96.67 ± 3.33	4.16 ± 0.047 ^{ab}	4.39 ± 0.87 ^{ab}	826.64 ± 151.31 ^{ab}
GA	100 ± 0.00	8.94 ± 0.44 ^c	5.06 ± 0.18 ^{ab}	1400.67 ± 61.10 ^c
PPL2	96.67 ± 3.33	4.27 ± 0.3 ^{ab}	5.08 ± 0.85 ^{ab}	904.26 ± 90.46 ^{ab}
PPL3	96.67 ± 3.33	3.79 ± 0.32 ^{ab}	3.44 ± 0.92 ^a	699.44 ± 137.54 ^a
PPL5	86.67 ± 6.67	4.46 ± 0.21 ^{ab}	5.98 ± 0.83 ^{ab}	904.29 ± 39.28 ^{ab}
PPL6	90.00 ± 5.77	5.27 ± 0.57 ^{ab}	7.52 ± 1.83 ^b	1151.4 ± 218.04 ^{abc}
PPL8	96.67 ± 3.33	3.59 ± 0.83 ^a	3.75 ± 1.24 ^a	709.53 ± 215.83 ^a
PPL9	93.33 ± 3.33	5.05 ± 0.8 ^{ab}	5.32 ± 0.84 ^{ab}	968.42 ± 122.60 ^{abc}
PPL10	93.33 ± 3.33	4.64 ± 0.48 ^{ab}	5.16 ± 1.69 ^{ab}	914.6 ± 232.48 ^{ab}
PPL11	93.33 ± 3.33	5.64 ± 0.12 ^b	7.82 ± 0.42 ^b	1256.13 ± 78.20 ^{bc}
PPL12	90.00 ± 5.77	5.60 ± 1.22 ^b	7.45 ± 0.31 ^b	1174.43 ± 71.33 ^{abc}
PPL14	96.67 ± 3.33	4.30 ± 0.26 ^{ab}	6.31 ± 0.63 ^{ab}	1025.13 ± 107.94 ^{abc}
PPL15	100 ± 0.00	4.76 ± 0.26 ^{ab}	6.53 ± 1.22 ^{ab}	1129.63 ± 146.88 ^{abc}
PPL18	96.67 ± 3.33	5.27 ± 0.21 ^{ab}	6.19 ± 0.38 ^{ab}	1107.37 ± 85.96 ^{abc}

dH₂O- Type 1 distilled water, IAA- 20 ppm indole-3-acetic acid, GA- 20 ppm gibberellic acid. *Seeds that did not germinate were not included in calculation of mean values.

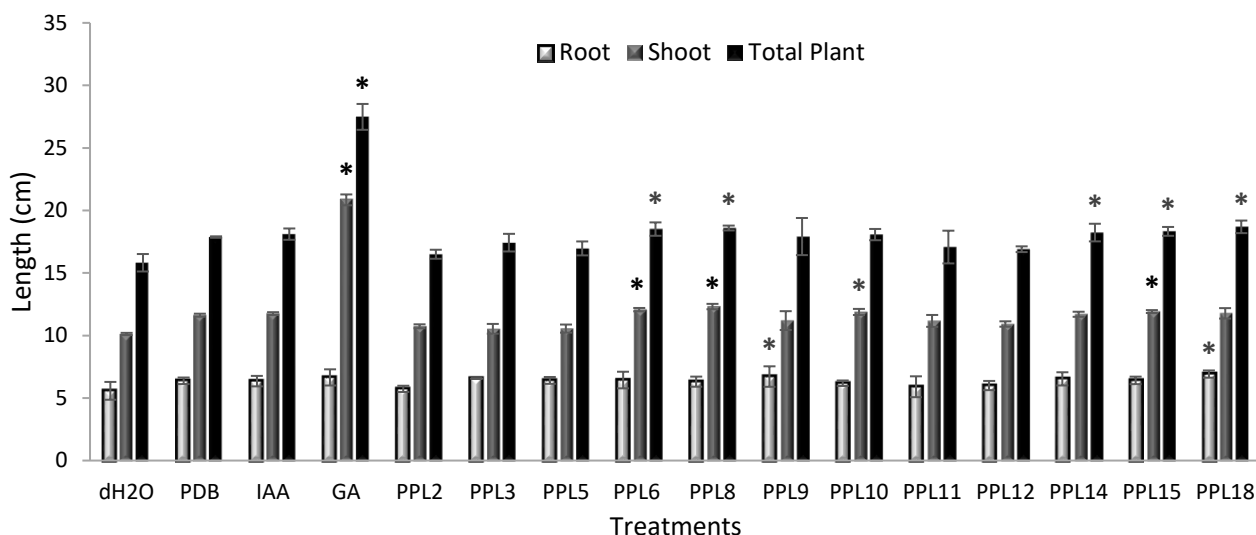


Fig. 3 – Effect of endophyte culture filtrates (CFs) on shoot, root and total length of PSB Rc10 rice seedlings 7 days after treatment. Data are means \pm SE, the experiment was conducted in three replicates of 5 seedlings each.

Effect on plant biomass

The GA-treated seedlings had the highest shoot dry weight averaging 7.30 mg while endophyte isolates *M. murinus* PPL10 and isolate PPL9 had significant results, 6.52 mg and 6.63 mg respectively (Fig. 4). The seedlings treated with *H. lixii* PPL8 CF have the heaviest roots of 12.74 mg. However, all the other treatment values were statistically comparable to this value. In terms of total plant biomass, all endophyte CF treatment values were not significantly different from the positive and negative controls. However, several isolates caused an observable increase in biomass particularly when compared to the positive control IAA and these isolates were *T. harzianum* PPL6, *H. lixii* PPL8, *A. versicolor* PPL15, and *M. murinus* PPL18.

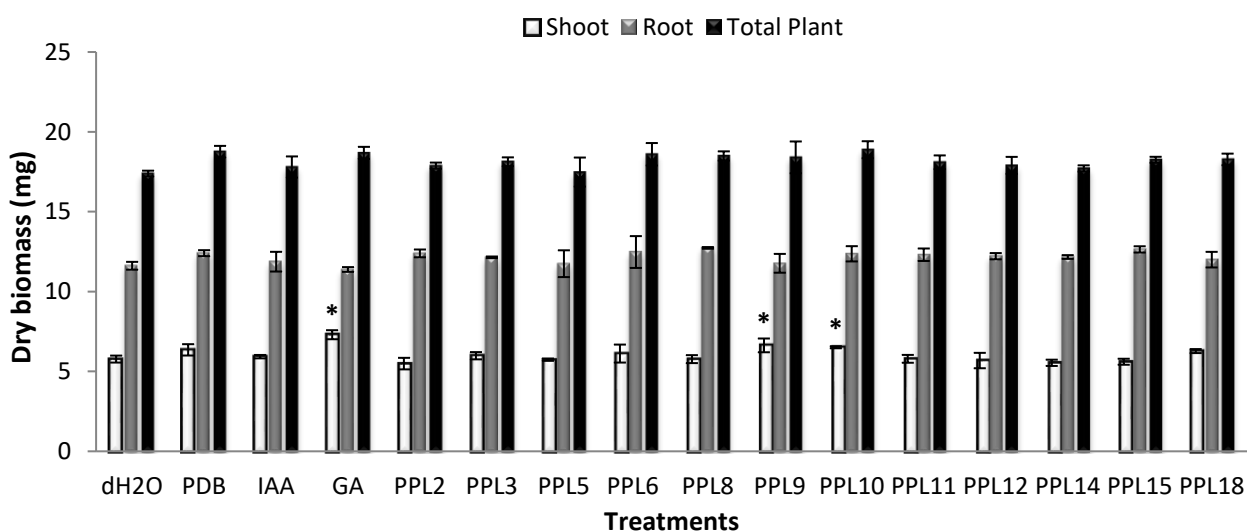


Fig. 4 – Effect of endophyte culture filtrates (CFs) on shoot, root and total plant dry biomass of PSB Rc10 rice seedlings 7 days after treatment. Data are means \pm SE, the experiment was conducted in three replicates of 5 seedlings each.

Discussion

The symbiotic association of fungi and plants is well-characterized. Most reported studies, however, focused on mycorrhizal fungi-plant associations. The growing studies on fungal endophytes show that all plants in their natural habitat harbor fungal endophytes and have functional roles that impact plant ecology and fitness (Yokoya et al. 2017). In the present study, 18 morphospecies of fungal endophytes were isolated from the roots of *Diket red*. All of these were filamentous fungi and no culturable yeasts were isolated.

Members of the genus *Scopulariopsis* have been documented as soil-inhabitants and common pathogens of animals (Sandoval-Denis et al. 2013). In this case, *Microascus murinus* in the soil might have infected *Diket red* rice and shifted from being a saprobe or parasite into a mutual symbiont. This research may be the first to report the plant growth-promoting traits of an endophytic *Microascus murinus* and study the effect of its metabolite on the growth of rice seedlings. The fungus *T. harzianum*, on the other hand, is an opportunistic plant symbiont widely used as a biofertilizer and biocontrol agent against phytopathogenic fungi (Lorito et al. 2010). It was previously isolated from plants like pigeon pea, maize, and sorghum (Zhao et al. 2013, Muvea et al. 2014). The other isolates are also previously reported as endophytes that provide different benefits to their plant hosts, i.e. *C. cladosporioides* in fir moss and sunflower, *A. versicolor* in red and green sea algae, and *Chaetosphaeria sp.* in sand couch-grass (Sánchez-Márquez et al. 2008, Zhang et al. 2011, Huwas et al. 2012, Liu et al. 2012, Waqas et al. 2013).

In-vitro screening of plant growth-promoting traits revealed that all 18 fungi morphospecies isolated were found to produce IAA in varying concentrations. The isolate PPL14 is the highest producer with 55.5 $\mu\text{g IAA ml}^{-1}$ comparable to the 52.2 $\mu\text{g IAA ml}^{-1}$ produced by a rhizosphere associated *T. harzianum* strain (Jogaiah et al. 2013). In a study by Redman et al. (2011), strains of endophytic fungi isolated from native plants in high stress environments that were capable of IAA production highly enhanced rice plant growth under salinity, drought, and temperature stress. Endophytic fungi strains also have been shown to promote the growth of various test plants such as *Solanum lycopersicum* and *Arabidopsis* via production of IAA (Sirrenberg et al. 2007, Khan et al. 2016).

Fungi have been reported to produce siderophores, which are iron-chelating compounds that sequester ferric iron for plant's use and also inhibit growth of phytopathogens by directly competing with them in iron resource sequestration (Calvente et al. 1999, Glick et al. 1999). Iron is an abundant mineral in the environment, but its availability to plant roots is limited (Morrissey & Guerinot 2009). The result showed that all fungal endophytes isolated have the ability to produce hydroxamate siderophores and therefore may play an important role in the growth of the rice plant in its natural environment by enhancing iron uptake.

Phosphate solubilization by different microorganisms provides a mechanism for the host plant to use this nutrient in its soluble form (Sharma et al. 2013). In order to solubilize inorganic phosphates, microorganisms produce organic acids that chelate the cations bound to inorganic phosphates leading to the release of the bound phosphates (Whitelaw et al. 1999). The release of organic acids explains the decrease in pH of the medium and the formation of yellow zones when phosphate solubilization activity is present. Fungal species belonging to the genera *Penicillium*, *Trichoderma*, *Fusarium*, *Aspergillus* among others, have been reported to have phosphate solubilizing activities (Wakelin et al. 2004, Barroso & Nahas 2005, Vitorino et al. 2012, Resende et al. 2014). In this study, four isolates were positive for phosphate solubilization (*C. cladosporioides*

PPL2 and PPL3, isolate PPL14, *A. versicolor* PPL15.). The Solubilization Index (SI) values of the four isolates were higher as compared to the SI values of some endophytic fungi strains tested by Vitorino et al. (2012) and Nutaratat et al. (2014). Moreover, the isolates *C. cladosporioides* PPL2 and PPL3, were also able to solubilize CaHPO_4 based from this study (Table 2).

The production of ammonia (NH_3) and catalase activity have been widely studied in characterizing plant growth-promoting bacteria (Joseph et al. 2007, Miliūtė & Buzaitė 2011), thus it is of interest that these two characteristics are tested for the isolated endophytes. NH_3 is a form readily available for biological processes, therefore endophytic microorganisms with the capability to fix

atmospheric N and produce NH₃ offers a way of enhancing N nutrition in plants (Yang et al. 2014). Samuel & Muthukkaruppan (2011) reported that rhizobacteria and fungi associated with rice, mangrove, and effluent contaminated soil have the capability to produce NH₃. It was demonstrated that all fungal endophyte isolates produced NH₃ as indicated by the formation of a blue color reaction. Isolates *A. versicolor* PPL5, *H. lixii* PPL8, *M. murina* PPL10, isolate PPL11, PPL14, and *M. murina* PPL18, as indicated by their intense blue color reaction, may have been important endophytes in giving available N to the plant host. Catalase, an important enzyme that combats the damaging oxidative stress caused by reactive oxygen species (ROS), was also tested in all of the 12 endophyte isolates. All isolates were positive for catalase activity, and therefore may have provided defense against oxidative damage in their host plant. *In vivo* studies showed that inoculation of fungal endophytes into plants increases the endogenous levels of catalase and other enzymes necessary in mitigating oxidative damage caused by ROS under heavy metal, drought, and salt stress (Khan et al. 2012b, Li et al. 2012, Khan et al. 2013).

The effects of the bioactive secondary metabolites produced by the endophytes in promoting seedling vigor and plant growth were assessed. The rice plant is widely used as an indicator test plant in screening bioassays since it is easy to grow in a simple water-agar media set-up under aseptic conditions (Khan et al. 2012b). The use of water-agar medium devoid of any nutrients and minerals, and treatment of the seeds with 20 ppm paclobutrazol would help in the determination of the sole effect of endophyte CF application in the promotion of rice growth. The endophyte isolates including *T. harzianum* PPL6, isolate PPL11, and *C. tulasneorum* PPL12 were notable for enhancing seedling vigor. *T. harzianum* had been reported to produce harzianolide, a secondary metabolite that can influence early plant development through enhancement of root length (Cai et al. 2013).

The three endophyte isolates, *M. murinus* PPL18, *T. harzianum* PPL6, and *H. lixii* PPL8, outperformed the other isolates in promoting overall plant growth. In terms of shoot growth, the isolates *H. lixii* PPL8 and *T. harzianum* PPL6 significantly increased shoot length of the rice seedlings while *M. murinus* PPL18 significantly increased root length. Based on the IAA assay, these three endophyte isolates produced substantial amounts of IAA which can directly promote plant growth. All of them were positive for siderophore production, NH₃ production, and catalase tests which can further augment plant growth. These endophyte isolates could be developed into environmentally-sustainable bio-inoculants that could help promote plant growth and enhance nutrient assimilation of agriculturally important crops.

Plant biomass is another important parameter in the study of plant growth since it is a basis for the calculation of net primary production and growth rate (Golzarian et al. 2011). All the treatments were found to be statistically comparable with each other, together with the effect of GA. On the other hand, some isolates such as *T. harzianum* PPL6, *H. lixii* PPL8, *A. versicolor* PPL15, and *M. murinus* PPL18 caused an increase in biomass when compared to the positive control IAA. The effect of combinatory treatments of fungal endophytes on plant growth and yield is a promising prospect for further studies.

Conclusion

The results from this study have provided insights into the endophytic fungi community associated with the roots of a traditional rice landrace in its natural environment. The endophytic isolates were demonstrated to have various plant growth-promoting traits such as IAA, siderophore, phosphate solubilization, NH₃, and catalase production activities. The application of CFs of the isolates PPL11 and *T. harzianum* PPL6 on rice seeds improved SVI while *H. lixii* PPL8, *T. harzianum* PPL6, *M. murinus* PPL18, *A. versicolor* PPL15, and isolate PPL14 on rice seedlings significantly promoted plant growth and dry biomass. These results provide support for the potential of endophytic fungi for agricultural purposes as plant growth promoters. Further studies are needed to determine the effectiveness and practicability of endophytic fungi inoculation on crops for field application.

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References

- Amprayn KO, Rose MT, Kecskes M, Pereg L et al. 2012 – Plant growth promoting characteristics of soil yeast (*Candida tropicalis* HY) and its effectiveness for promoting rice growth. *Applied Soil Ecology* 61, 295–299.
- Barroso CB, Nahas E. 2005 – The status of soil phosphate fractions and the ability of fungi to dissolve hardly soluble phosphates. *Applied Soil Ecology* 29, 73–83.
- Cai F, Yu G, Wang P, Wei Z et al. 2013 – Harzianolide, a novel plant growth regulator and systemic resistance elicitor from *Trichoderma harzianum*. *Plant Physiology and Biochemistry* 73, 106–113.
- Calvente V, Benuzzi D, Tosetti MIS. 1999 – Antagonistic action of siderophores from *Rhodotorula glutinis* upon the postharvest pathogen *Penicillium expansum*. *International Biodeterioration and Biodegradation*, 43 167–172.
- Chang TT, Vergara BS. 1975 – Varietal diversity and morpho-agronomic characteristics of upland rice, in: *Major Research in Upland Rice*. International Rice Research Institute, Los Baños, Philippines 72–90.
- Glick BR, Patten CL, Holguin G, Penrose DM. 1999 – *Biochemical and Genetic Mechanisms Used by Plant Growth Promoting Bacteria*. Imperial College Press, London.
- Glickmann E, Dessaux Y. 1995 – A critical examination of the specificity of the salkowski reagent for indolic compounds produced by phytopathogenic bacteria. *Applied and Environmental Microbiology* 61, 793–796.
- Golzarian M, Frick R, Rajendran K, Berger B et al. 2011 – Accurate inference of shoot biomass from high-throughput images of cereal plants. *Plant Methods* 7: 2. Doi: 10.1186/1746-4811-7-2.
- Hamayun H, Khan SA, Khan, AL, Ahmad A et al. 2011 – Gibberellin producing *Neosartorya* sp. CC8 reprograms chinese cabbage to higher growth. *Scientia Horticulturae* 129, 347–352.
- Hucker GJ, Wall WA. 1922 – The use of agar slants in detecting ammonia production and its relation to the reduction of nitrates. *Journal of Bacteriology* 7, 513–516.
- Huwas UW, El-Beih AA, El-Halawany AM. 2012 – Bioactive anthraquinones from endophytic fungus *Aspergillus versicolor* isolated from red sea algae. *Archives of Pharmacal Research* 35(10), 1749–56.
- Jogaiah S, Mostafa M, Phan Tran L, Ito S. 2013 – Characterization of rhizosphere fungi that mediate resistance in tomato against bacterial wilt disease. *Journal of Experimental Botany* 64(12), 3829–3842.
- Joseph B, Patra RR, Lawrence R. 2007 – Characterization of plant growth promoting rhizobacteria associated with chickpea (*Cicer arietinum* L.). *International Journal of Plant Production* 2, 141–152.
- Khan AL, Halo BA, Elyassi A, Ali S et al. 2016 – Indole acetic acid and ACC deaminase from endophytic bacteria improves the growth of *Solanum lycopersicum*. *Electronic Journal of Biotechnology* 21, 58–64.
- Khan SA, Hamayun M, Khan AL, Lee IJ et al. 2012a – Isolation of Plant Growth Promoting Endophytic Fungi from Dicots Inhabiting Coastal Sand Dunes of Korea. *Pakistan Journal of Botany* 44(4), 1453–1460.
- Khan AL, Hamayun M, Kang S, Kim Y et al. 2012b – Endophytic fungal association via gibberellins and indole acetic acid can improve plant growth under abiotic stress: an example of *Paecilomyces formosus* LHL 10. *BMC Microbiology* 12, 3. Doi: 10.1186/1471-2180-12-3.
- Khan AL, Waqas M, Hamayun M, Al-Harrasi A et al. 2013 – Co-synergism of endophyte *Penicillium resedanum* LK6 with salicylic acid helped *Capsicum annuum* in biomass recovery and osmotic stress mitigation. *BMC Microbiology*. 13(51). <https://doi.org/10.1186/1471-2180-13-51>

- Krings M, Taylor TN, Hass H, Kerp H et al. 2007 – Fungal endophytes in a 400-million-yr-old land plant: infection pathways, spatial distribution, and host responses. *New Phytologist* 174, 648–657.
- Lahlali R, McGregor L, Song T, Gossen BD et al. 2014 – *Heteroconium chaetospora* Induces Resistance to Clubroot via Upregulation of Host Genes Involved in Jasmonic Acid, Ethylene, and Auxin Biosynthesis. *PLoS ONE* 9(4), e94144. Doi: 10.1371/journal.pone.0094144.
- Li X, Bua N, Li Y, Maa L et al. 2012 – Growth, photosynthesis and antioxidant responses of endophyte infected and non-infected rice under lead stress conditions. *Journal of Hazardous Materials* 213, 55–61.
- Liu XH, Miao FP, Li XD, Yin L, Ji NY. 2012 – A new sesquiterpene from an endophytic *Aspergillus versicolor* strain. *Natural Product Communications* 7(7), 819–820.
- Lorito M, Woo SL, Harman GE, Monte E. 2010 – Translational research on *Trichoderma*: from ‘omics to the field. *Annual Review of Phytopathology* 48, 395–417.
- Mariño CG, Cabrera R, Reina M, Gonzales-Coloma A. 2007 – Fungal Endophytes and their Role in Plant Protection. *Current Organic Chemistry*. 11(8): 707–720. Doi: 10.2174/138527207780598765
- Miliūtė I, Buzaitė O. 2011 – IAA production and other plant growth promoting traits of endophytic bacteria from apple tree. *Biologija* 57(2), 98–102.
- Morrissey J, Guerinot ML. 2009 – Iron uptake and transport in plants: The good, the bad, and the ionome. *Chemical Reviews* 109(10), 4553–4567.
- Muvea AM, Meyhofer R, Subramanian S, Poehling HM et al. 2014 – Colonization of onions by endophytic fungi and their impacts on the biology of *Thrips tabaci*. *PLoS One* 9(9), e108242. Doi: 10.1371/journal.pone.0108242.
- Neilands JB. 1981 – Microbial iron compounds. *Annu. Rev. Biochem.* 50, 715.
- Nutaratat P, Srisuk N, Arunrattiyakorn P, Limtong S. 2014 – Plant growth-promoting traits of epiphytic and endophytic yeasts isolated from rice and sugar cane leaves in Thailand. *Fungal Biology* 118, 683–694.
- Rai M, Rathod D, Agarkar G, Dar M et al. 2014 – Fungal growth promotor endophytes: a pragmatic approach towards sustainable food and agriculture. *Symbiosis* 62, 63–79.
- Ram SG, Thiruvengadam V, Vinod KK. 2007 – Genetic diversity among cultivars, landraces and wild relatives of rice as revealed by microsatellite markers. *Journal of Applied Genetics* 48(4), 337–345.
- Redman RS, Kim YO, Woodward CJDA, Greer C et al. 2011 – Increased fitness of rice plants to abiotic stress via habitat adapted symbiosis: a strategy for mitigating impacts of climate change. *Plos One* 6(7), e14823. Doi: 10.1371/journal.pone.0014823.
- Resende MP, Jakoby ICMC, dos Santos LCR, Soares MA et al. 2014 – Phosphate solubilization and phytohormone production of endophytic and rhizosphere *Trichoderma* isolates of guanandi (*Calophyllum brasiliense* Cambess). *African Journal of Microbiology Research* 8(27), 2616–2623.
- Rodriguez RJ, Redman RS, Henson JM. 2004 – The Role of Fungal Symbioses in the Adaptation of Plants to High Stress Environments. *Mitigation and Adaptation Strategies for Global Change* 9, 261–272.
- Rodriguez RJ, Henson J, Van Volkenburgh E, Hoy M et al. 2008 – Stress tolerance in plants via habitat-adapted symbiosis. *The ISME Journal* 12, 404–416.
- Samson RA, Klopotek A. 1972 – *Scopulariopsis murina*, a new fungus from self-heated compost. *Archives of Microbiology* 85(2), 175–180.
- Samuel S, Muthukkaruppan SM. 2011 – Characterization of plant growth promoting rhizobacteria and fungi associated with rice, mangrove and effluent contaminated soil. *Current Botany* 2(3), 22–25.
- Sánchez-Márquez S, Bills GF, Zabalgogea I. 2008 – Diversity and structure of the fungal endophytic assemblages from two sympatric coastal grasses. *Fungal Diversity*, 33, 87–100.

- Sandoval-Denis M, Gené J, Sutton DA. 2016 – Redefining *Microascus*, *Scopulariopsis* and allied genera. *Persoonia* 36, 1–36.
- Sandoval-Denis M, Sutton DA, Fothergill AW, Cano-Lira J et al. 2013 – *Scopulariopsis*, a poorly known opportunistic fungus: spectrum of species in clinical samples and in vitro responses to antifungal drugs. *Journal of Clinical Microbiology* 51, 3937–3943.
- Schulz B, Boyle C. 2005 – The endophytic continuum. *Mycological Research* 109, 661–686.
- Sharma SB, Sayyed RZ, Trivedi MH, Gobi TA. 2013 – Phosphate solubilizing microbes: sustainable approach for managing phosphorus deficiency in agricultural soils. *SpringerPlus* 2: 587.
- Sirrenberg A, Gobel C, Grond S, Czempinski N et al. 2007 – *Piriformospora indica* affects plant growth by auxin production. *Physiologia Plantarum* 131, 581–589.
- Strobel G, Daisy B. 2003 – Bioprospecting for microbial endophytes and their natural products. *Microbiology and Molecular Biology Reviews* 67(4), 491–502.
- Tamura K, Stecher G, Peterson D, Filipinski A, Kumar S. 2013 – MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Molecular Biology and Evolution* 30(12), 2725–2729.
- Vala AK, Dave BP, Dube HC. 2006 – Chemical characterization and quantification of siderophores produced by marine and terrestrial aspergilli. *Canadian Journal of Microbiology* 52, 603–607.
- Vitorino LC, Silva FG, Soares MA, Souchie EL et al. 2012 – Solubilization of calcium and iron phosphate and in vitro production of indoleacetic acid by endophytic isolates of *Hyptis marrubioides* Epling (Lamiaceae). *International Research Journal of Biotechnology* 3(4), 47–54.
- Wakelin SA, Warren RA, Harvey PR, Ryder MH. 2004 – Phosphate solubilization by *Penicillium* spp. closely associated with wheat roots. *Biology and Fertility of Soils* 40, 36–43.
- Waqas M, Khan AL, Ali L, Kang SM et al. 2013 – Seed germination-influencing bioactive secondary metabolites secreted by the endophyte *Cladosporium cladosporioides* LWL5. *Molecules* 18, 15519–15530.
- Waqas M, Khan AL, Kamran M, Hamayun M et al. 2012 – Endophytic fungi produce gibberellins and indoleacetic acid and promotes host-plant growth during stress. *Molecules* 17, 10754–10773.
- Whitelaw MA, Harden TJ, Helyar KR. 1999 – Phosphate solubilization in solution culture by the soil fungus *Penicillium radicum*. *Soil Biology and Biochemistry* 31, 655–665.
- Yang B, Ma HY, Wang XM, Jia Y et al. 2014 – Improvement of nitrogen accumulation and metabolism in rice (*Oryza sativa* L.) by the endophyte *Phomopsis liquidambari*. *Plant Physiology and Biochemistry* 82, 172–182.
- Yokoya K, Postel S, Fang R, Sarasan V. 2017 – Endophytic fungal diversity of *Fragaria vesca*, a crop wild relative of strawberry, along environmental gradients within a small geographical area. *Peer J* 5, e2860.
- Zhang ZB, Zeng QG, Yan RM, Wang Y et al. 2011 – Endophytic fungus *Cladosporium cladosporioides* LF70 from *Huperzia serrata* produces Huperzine A. *World Journal of Microbiology & Biotechnology* 27, 479–486.
- Zhao J, Li C, Wang W, Zhao C et al. 2013 – *Hypocrea lixii*, novel endophytic fungi producing anticancer agent cajanol, isolated from pigeon pea (*Cajanus cajan* [L.] Millsp.). *Journal of Applied Microbiology* 115(1), 102–113.